Acetyl-L-carnitine and α-lipoic acid supplementation of aged beagle dogs improves learning in two landmark discrimination tests

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ABSTRACT **Beagle dogs between 7.6 and 8.8 years of age administered a twice daily supplement of α-lipoic acid (LA) and acetyl-L-carnitine (ALC) over ~2 months made significantly fewer errors in reaching the learning criterion on two landmark discrimination tasks compared to controls administered a methylcellulose placebo. Testing started after a 5 day wash-in. The dogs were also tested on a variable delay version of a previously acquired spatial memory task; results were not significant. The improved performance on the landmark task of dogs supplemented with LA + ALC provides evidence of the effectiveness of this supplement in improving discrimination and allocentric spatial learning. We suggest that long-term maintenance on LA and ALC may be effective in attenuating age-associated cognitive decline by slowing the rate of mitochondrial decay and cellular aging.—Milgram, N. W., Araujo, J. A., Hagen, T. M., Treadwell, B. V., Ames, B. N. Acetyl-L-carnitine and α-lipoic acid supplementation of aged beagle dogs improves learning in two landmark discrimination tests. *FASEB J.* 21, 3756–3762 (2007)**

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AGING IS ASSOCIATED WITH A DECLINE in the vitality of cells, tissues, and organs (1–3). Aging is also associated with behavioral changes, including cognitive decline and age-related disorders associated with brain pathology (2, 3). At the cellular level, much of the aging process is believed to be a consequence of oxidative damage to mitochondria, which decay with age (1, 2, 4–12).

One strategy for delaying the mitochondrial decay of aging consists of treatment with a combination of α-lipoic acid (LA) and acetyl-L-carnitine (ALC; refs. 4–8, 10–12). Both compounds function in the mitochondria as cofactors required for the production of energy from fat and carbohydrates (4, 5). Maintenance of aged rats on a diet containing LA and ALC has been shown to have several beneficial effects on normal characteristics of aging, including increasing physical activity (10), reducing oxidative damage (12), improving mitochondrial function (10), and improving water maze learning (11).

The present experiment sought to further explore the utility of this strategy in a canine model of cognitive aging (13–16). Three different cognitive tests were used: two landmark discrimination tests and a variable-delay nonmatching-to-position (DNMP) test. We have previously shown that certain tasks in aged dogs provide an animal model of age-associated cognitive decline (17–22). Aged dogs also provide a model for region-specific β-amyloid pathology, which is seen in both successful aging and Alzheimer's disease (23–25). Furthermore, we have shown that aged dogs exhibit increased levels of oxidative stress (26).

The landmark discrimination task was first described for use with dogs by Milgram *et al.* (18). The test protocol comprises at least two tasks. The first task, referred to as land-0, assesses general discrimination learning ability and requires subjects to discriminate between two coasters; the correct one has a landmark (a yellow wooden peg) attached to the center. In the second task, land-1, the landmark is placed 1 cm away from one or the other coaster, and the subjects are required to approach the coaster closest to the landmark. Land-1 provides a measure of allocentric spatial ability, which involves the utilization of an external landmark as a spatial referent. Allocentric spatial function is distinguished from egocentric spatial orientation, which utilizes as a reference the position of the organism in space (17). The rationale for selection of the landmark task is threefold. First, human and nonhuman primate cognitive aging and dementia are associated with deficits in visuospatial function in general

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and allocentric spatial learning in particular (27–30). Allocentric deficits are an early sign of human dementia (27, 30). Young monkeys use external cues, and hence allocentric strategies, to solve a primate version of the radial arm maze task; aged monkeys used a different response sequencing strategy (29). Second, in canines the landmark discrimination protocols show marked age sensitivity (18, 19). In a previous experiment, for example, only two-thirds of a group of old animals (8–12 years) were able to learn the Land-1 task and only one-third was able to learn a more complex version with the landmark 2 cm from the coaster. By contrast, every young animal (1.95–4.5 years) was able to learn both land-0 and land-1, and two-thirds of the young animals also learned the more complex version (19). Third, Milgram *et al.* (19) have previously tested aged beagle dogs maintained on a specially formulated antioxidant enriched diet that included DL-α-tocopherol acetate (1550 ppm), taurine (1095 ppm), ascorbic acid (100 ppm), and 1% inclusions of each of the following (1-to-1 exchange for corn): spinach flakes, tomato pomace, grape pomace, carrot granules, citrus pulp, and one-fourth the amount (compared to the present study) of LA and L-carnitine. That study (19) showed improved performance relative to controls on a landmark discrimination learning task within one month of the start of treatment. The present study employed only LA and ALC (rather than L-carnitine used previously; ref. 19) and did not include the other components tested previously in the antioxidant enriched diet.

The DNMP task was first described by Head *et al.* (31, 32). The present study used a variable-delay DNMP protocol in which highly practiced dogs' ability to remember the location of a reward was tested over short (5 s), medium (55 s), and long (105 s) time intervals. The DNMP task was included to assess the effect of the treatment strategy on visuospatial memory. This task models working memory decline in both human aging and dementia (33). Dogs show marked age-dependent deficits in learning and memory on the DNMP task (31), which can be detected as early as 6 years of age (16). Furthermore, performance varies as a function of delay in dogs that have acquired the task (34). Similar age-dependent deficits in spatial memory occur in both nonhuman and human primates (2, 28, 35, 36).

MATERIALS AND METHODS

Subjects

The subjects were random-source beagle dogs of both sexes between 7.6 and 8.8 years of age from the colony of CanCog Technologies. All subjects were test-sophisticated and had previously been trained on a variable delay version of a DNMP. Before the start of this study, all subjects were administered the DNMP task over five consecutive sessions and ranked. The best and worst performing animals were assigned to one group, and the second best and second worst performing animals were assigned to a second group. The remaining animals were assigned in a quasi-random fashion such that there were three males and three females in each group and the average ages did not differ significantly. One group was then assigned by coin toss to the treatment condition and the other to the control condition. The group assignments and relevant grouping variables are shown in **Table 1**.

Housing and behavioral enrichment

The subjects were housed in stainless steel pens, either 2.5′ × 16′ or 5′ × 16′, with 2′ × 4′ perches. The floors were epoxy painted and heated, and the exterior walls had windows ~10′ from ground level that provide natural lighting. The subjects were group-housed in compatible groups (3 or 4 to a pen) whenever possible and were given daily outdoor exercise for approximately half an hour per day. The pens were cleaned daily with a power washer, and the housing area was disinfected regularly with a sanitizing agent.

ALC + LA supplement

The ALC + LA supplement consisted of a powder provided by Juvenon. Daily oral doses were 11.0 mg/kg (LA) and 27.5 mg/kg (ALC), provided in capsules placed in meatballs consisting of Hill's Prescription Diet® (P/D). This dose was similar, on a per kilogram body weight basis, to that previously used in human studies without significant side effects (37–41). The protocol was initiated with a 5 day washin phase, during which the subjects received no behavioral testing. Subjects were maintained on the test compound during the entire testing period. Each animal was dosed twice each day at ~8 am and ~3:45 pm. The first dosing was 2–3 h before cognitive testing, with each capsule containing half the total daily dose. The control group received equivalent amounts of methylcellulose. All phases of the study were blinded: different individuals prepared and administered the capsules; a third group of individuals conducted the cognitive testing and were blinded as to the treatment status of the animals.

Test apparatus and software

The test apparatus, a wooden box (~3′×5; details will be provided on request), was a modified version of the Wisconsin General Test Apparatus originally used in testing nonhuman primates and subsequently modified for use with dogs (20). The front consisted of parallel rows of stainless steel bars of adjustable heights that provided three gates through which a dog was able to respond to objects presented to it on a tray. The experimenter was separated from the dog by a partition containing a one-way mirror and a hinged-door that when opened allows the tray to be presented to the dog. The tray contained one medial and two lateral food wells.

All cognitive testing used the computer program DogCog (CanCog Technologies). The program was under administrator control, which prevented the cognitive test technicians from modifying any of the test parameters. The DogCog program also randomly calculated all test sequences and controlled all timing, intertrial intervals, inspection intervals, and delays. The experimenter used a key press to enter each response, which was recorded by the software.

TABLE 1. *Characteristics of dogs used in study*

Subject	Sex	Date of birth	Age at start	Weight at start (kg)	Start date	Baseline (DNMP)
			Methylcellulose			
Chilli	F	7/21/1998	7.8	11.8	4/21/2006	0.7
Banjo	M	6/21/1997	8.8	17.7	4/21/2006	0.689
Randy	M	6/21/1998	7.8	18.6	4/21/2006	0.639
Donnie	M	6/21/1998	7.8	10.8	4/21/2006	0.756
Cherry	F	10/8/1997	8.5	9.2	4/21/2006	0.756
Waggles	F	7/11/1998	7.8	10.3	4/21/2006	0.778
Mean			8.08333	13.0667		0.71967
			Lipoic Acid and ALCAR			
Subject	Sex	Date of birth	Age		Start date	(DNMP)
Bubbles	F	9/27/1998	7.6	8.4	4/21/2006	0.711
Barry	M	6/21/1998	7.8	16.2	4/21/2006	0.722
Miami	F	8/19/1997	8.7	8.1	4/21/2006	0.678
Joey	M	6/21/1997	8.8	13.2	4/21/2006	0.856
Debbie	F	8/20/1997	8.7	9.7	4/21/2006	0.578
Steve	M	7/11/1998	7.8	12.5	4/21/2006	0.733
Mean			8.22222	11.3500		0.713

Baseline DNMP score represents mean ratio of correct to total responses for each subject. Thus, the ALC + LA group, on average, performed slightly more poorly than controls.

Behavioral testing protocols

Landmark discrimination test

This test involved two tasks, land-0 and land-1, and was conducted over a maximum of 55 days. Subjects were trained to approach one of two objects (white coasters) based on the proximity of the object to an external landmark (a yellow wooden peg (2 cm×2 cm×9 cm).

1. Land-0 discrimination task. In the land-0 task, the yellow peg was attached to the center of one of the two white coasters. A food reward, ~1 g of wet dog food (P/D), was placed in either the left or right food well. A white coaster with a yellow peg attached was then placed covering the reward. Food reward was stuck to the bottom of the nonrewarded coaster covering the other well to prevent the dogs from using odor cues. The door was raised, and the tray was moved to ~25 cm from the dog for a brief inspection interval. The tray was then presented to the dog, and the dog was given up to 30 s to respond by displacing one of the two coasters, with the correct coaster being the one with the attached yellow peg. The location of the peg was determined randomly by the computer, with the constraint that each side be correct on half of the trials of each test session. In each session, a partial correction procedure was used, in which a dog was permitted to correct its response without the tray being removed after its first incorrect try only.

In Phase One, each dog was allowed a maximum of 10 test sessions to reach a criterion level of performance. One session was conducted each day; each session consisted of 10 trials, with an intertrial interval of 30 s. A two-stage learning criterion was used. To pass the first stage, the dog had to respond correctly on at least 9 of 10 trials, or on 8 of 10 trials over 2 consecutive days, and to have no response failures (failure to respond within 30 s). The second-stage criterion required the subject to respond correctly on at least 70% of the next 30 trials over 3 consecutive sessions, without any response failures. A correct response was assigned a score of 1 and an incorrect response a score of 0. Response failures were assigned a score of 0.5, which is what their score would have been had they responded randomly, and were given an extra day of testing to complete the second criterion phase: an average score of 70% over all test days is required to pass the second stage. Testing was stopped in subjects once they passed the learning criterion, and they were given an error score equal to the total number of errors made over the entire test period.

Phase 2 consisted of a program of remedial training for subjects that did not pass the second stage criterion within the first 10 sessions. This phase occurred over 5 additional daily sessions, with 15 trials per day. At the start of the remedial training, the animals were presented with a single rewarded stimulus on the majority of the trials. With continued testing, more paired stimulus presentations were given.

In Phase 3, dogs that required remedial training underwent a maximum of 10 additional training sessions using the Phase 1 protocol.

At the completion of testing, dogs that reached criterion in Phase 1 were assigned an errors-to-criterion learning score. Dogs that did not reach the Phase 1 criterion in 10 sessions and underwent remedial training were assigned a score that was the sum of errors made during Phase 1 and errors made before reaching the Phase 3 criterion. For those animals that did not reach the Phase 1 criterion even after remedial training, the error score assigned was the total number of incorrect responses over both Phase 1 and Phase 3. Thus, all animals were assigned an error score, which was equal to the total number of errors made until they either passed the criterion level of performance or completed Phase 3.

2. Land-1 discrimination task. Training on land-1 started on the day after the subjects completed testing on land-0. On this task, the yellow peg was moved 1 cm medially and diagonally away from the edge of the coaster. The peg was attached to the food tray by a black piece of 2 cm wide Velcro, attached to both the base of the peg and the surface of the food tray. Each dog was allowed up to 25 consecutive daily training sessions to successfully complete the same two-stage learning

criteria used in land-0. In all other respects, the training procedures were identical to those followed in land-0.

DNMP task

Testing began 2 days after completing testing on land-1 and continued for 14 test sessions on consecutive days. The DNMP task utilizes visuospatial working memory to establish the correct location of a food reward. Each trial consisted of 2 presentations, a sample presentation and a test presentation.

The subject was presented with a single red block covering one of three food wells and allowed up to 30 s to displace the block and retrieve the food reward. After the sample presentation, a delay interval was initiated. The test component immediately followed the delay and involved presentation of two identical blocks, one of which was at the sample position and the other was at a one of the other two locations. Food reward was available only if the subject responded to the block at the new location. For each test session, delays of 5, 55, and 105 s were equally divided among the 18 trials; thus, on each test, there were exactly 6 trials at each delay. The intertrial interval was 30 s. The sequence of delays was randomized with the constraint that no delay interval could occur over more than three successive sessions.

RESULTS

In **Fig. 1**, total errors averaged for the test and control groups are plotted as a function of test session for the first 10 sessions of the land-0 phase and the first 25 sessions of the land-1 phase (for the land-0 phase, by session 10, 4 out of the 6 subjects in the LA+ALC group and only 2 of the 6 subjects in the control group had reached criterion). After completing remedial training (15 trials per day for 5 days, in which a 2nd coaster was not added), each subject learned to approach a single coaster), and one additional control animal and both LA + ALC animals reached criterion, although up to 10 additional training sessions (15 trials per day for 10 days) were required. As shown in Fig. 1, the land-1 test was more difficult. Five of six subjects in the LA + ALC group successfully passed the task, but only three of six animals in the control group were successful, despite having up 25 training sessions.

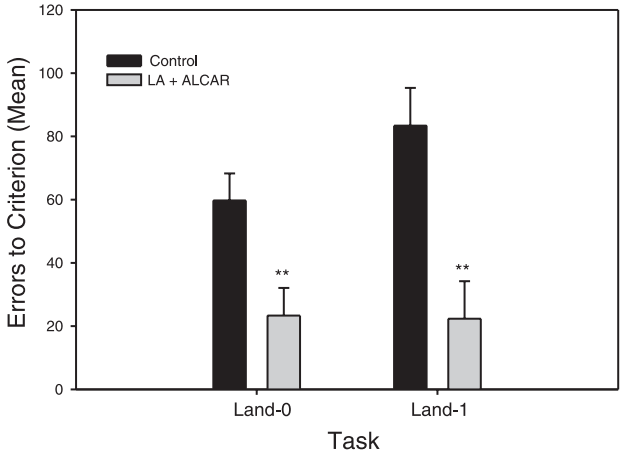

Figure 1. Errors to criterion is plotted as a function of treatment group (control *vs.* LA and ALC) and task (land-0 and land-1). $^{**}P < 0.01$

A repeated measures analysis of variance, with task (land-0 and land-1) as a within subject measure and treatment (test compound *vs.* control compound) as a between subject measure, indicated a significant treatment effect [$f\,(1,10)=13.77$; $P=0.004$], and a marginally significant interaction between task and treatment [$f\,(1,10)=3.41$; $P=0.0945$]. There were no other significant main effects or interactions. A separate analysis revealed no effects of sex. Univariate ANOVAs indicated significant treatment effects on both land-0 and land-1 ($P=0.012$ and $P=0.005$, respectively). As shown in Fig. 1, the group treated with LA + ALC made significantly fewer errors reaching criterion on both tasks than did the control group.

Results obtained on the DNMP task are summarized in **Fig. 2**. A repeated measures ANOVA with delay (5, 55, and 105 s) and test week (week 1 *vs.* week 2) as within subject variables and treatment (control *vs.* LA+ALC) as a between subject variable indicated a significant effect of delay interval ($P=<0.001$) but no other significant main effects or interactions. The absence of a significant difference between weeks 1 and 2 is indicative of the stability of performance on the task, although both groups showed reduced errors in the second week, when compared to the first.

DISCUSSION

The present results demonstrate that treatment of aged dogs with a combination of LA + ALC significantly improves performance on two landmark discrimination tasks intended to assess complex object discrimination learning and allocentric spatial learning. The dogs were rewarded in the first task, land-0, for approaching an object consisting of a coaster with an attached wooden peg and avoiding a coaster lacking the peg. As discussed previously, this task depends on object discrimination learning, with the coaster plus landmark constituting a discrete object that is associated with a reward (18). By contrast, the second task, land-1, depends on allocentric discrimination learning because the landmark does not come in contact with the coaster and because the stimulus configuration produced by the landmark plus coaster differed, depending on whether the correct response was to the right or left food well. The group differences in learning both tasks were statistically significant ($P<0.01$; Fig. 1).

After completion of the landmark phase of the study, the dogs were tested on a variable delay DNMP task to examine the effect of LA + ALC on visuospatial working memory. Although animals in the treatment group appeared to perform slightly better than the control group, results did not approach statistical significance (Fig. 2) nor did the groups differ statistically when the analysis took into consideration baseline performance (data not shown).

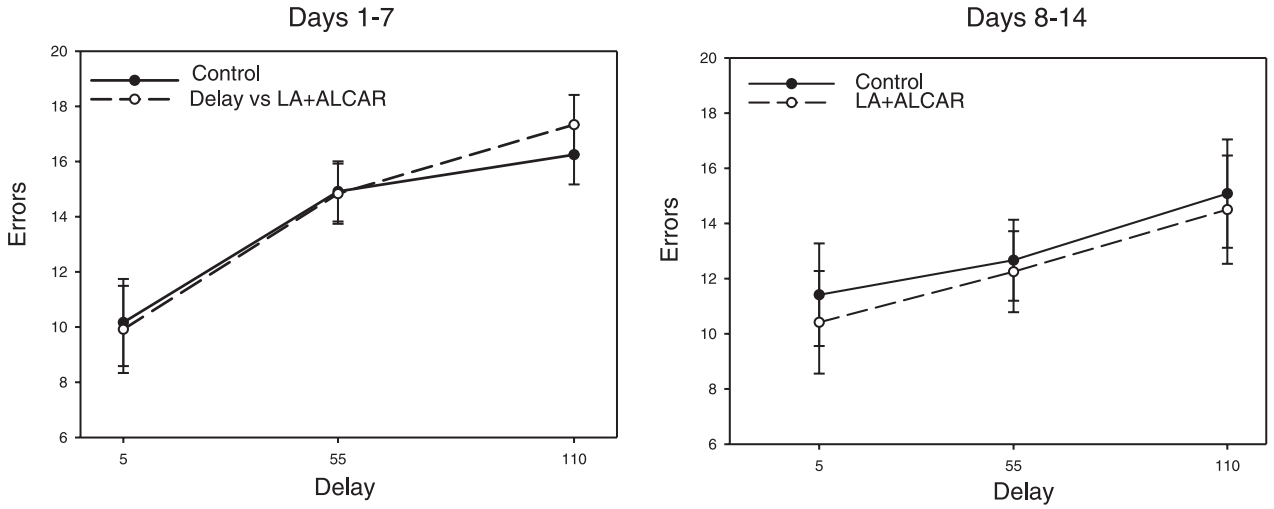

Figure 2. Performance as a function of delay for each of the 2 groups over the first (left) and second (right) test weeks.

The lack of effect of supplementation on the DNMP task could suggest that the cognitive-enhancing effectiveness of short-term maintenance on LA + ALC is task dependent. In this instance, the DNMP and the landmark tasks involve separate processes: performance on the DNMP task relies more on short-term memory, while the landmark discrimination tasks are dependent on rule learning and the ability to store information in long-term memory. However, an alternative explanation is that the animals had been trained and were well practiced on the DNMP task before the start of the study. This raises the possibility that their performance on that task was already optimal, and improvements due to LA + ALC supplementation may have been difficult to detect.

It is also possible that a longer period of LA + ALC supplementation may be required for an effect in the DNMP task. We previously observed that supplementation with an antioxidant cocktail did not affect performance on a DNMP task after 1 year of treatment but did lead to improved performance after 2 years (Milgram *et al.,* unpublished observations).

Therapeutic effectiveness

The superior performance of the dogs supplemented with the combination of LA + ALC provides evidence of its therapeutic effectiveness in reversing age-associated deficits in learning two different but interrelated tasks. This is the first study with dogs to demonstrate positive effects of the test combination, but the results are consistent with several previous studies done with rodents that indicate positive effects of either LA or ALC examined individually (42–45). Only one rodent study (11) examined the two compounds in combination. The results suggested global cognitive improvement on all tests, including water maze learning, memory of previous platform location, visible platform learning, and temporal memory (11). The present results are also consistent with a previous study in which dogs were administered a cocktail of many antioxidants that included small doses of LA and L-carnitine (19). The subjects on the antioxidant cocktail, when compared with controls, showed improved learning on both landmark and oddity discrimination learning protocols (19). The present results, however, provided a more robust effect, as the previous study reported a statistically significant effect for land-0 but not land-1.

The more efficient learning on the landmark tests observed in the LA + ALC compared to control groups may be due to greater mental energy caused by the supplement. A variety of mental functions, including learning, are known to be affected by the state of mental energy (46). Mitochondria supply almost all of the energy in the cell in the form of ATP, and the demonstrated primary effect of LA + ALC supplementation is to enhance mitochondrial function (4, 5, 10–12). For example, LA + ALC has previously been demonstrated to reverse biochemical markers of mitochondrial decay (10–12), which accompanies aging, and to improve spatial learning in aged rats (11). A second factor that could also contribute to improved learning is increased synthesis of the neurotransmitter acetylcholine in the supplemented group. Aged rats treated with ACL show improved cholinergic function (47), and *in vitro* studies have reported an increase in acetylcholine synthesis in neurons incubated in the presence of ACL (48).

Performance on the DNMP task, by contrast, was most likely influenced by previous learning (the animals were highly practiced on the task before the study), which could explain the lack of effect observed here. It is possible that LA + ALC could facilitate initial learning of the DNMP task.

In conclusion, the present results demonstrate that aged beagle dogs maintained on a diet supplemented with LA + ALC show significantly improved learning compared to controls on two landmark discrimination tasks, both of which show age sensitivity. By contrast, LA + ALC supplementation did not affect performance on

a delayed-matching-to-place spatial memory task. The effects of LA + ALC treatment over the several month duration of this study are most likely attributable to their effects in improving mental energy by enhancing mitochondrial function (4–12), although increased acetylcholine synthesis may also be a factor. We suggest that longer term treatment with LA + ALC is likely to have additional cognitive benefits, slowing or delaying the development of cognitive decline. FJ

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